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Communication

Measurement of long range H,C couplings in natural products in orienting media: a tool for structure elucidation of natural products

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Abstract

In this paper we show that water insoluble compounds dissolved in poly-γ-benzyl-glutamate are amenable to the measurement of a number of homo- and heteronuclear dipolar couplings. The sensitivity and experimental precision of dipolar couplings are sufficient to obtain a good match with the structure. In order to achieve the necessary precision for H,C dipolar couplings between protons and carbons that are not directly bound a new method for the measurement of heteronuclear long range couplings is introduced that allows a one-parameter fit to a HSQC-based experiment as reference experiment. The methodology is applied to menthol (1R, 3S, 4R).

Recently, Courtieu and co-workers have introduced carbon and deuterium-detected NMR spectroscopy of organic compounds dissolved in poly-γ-benzyl-L-glutamate (PBLG) and chloroform in order to distinguish enantiomers [1]. It could also been shown that in small organic molecules C,C dipolar couplings can be measured by the INADEQUATE approach in the same medium [2] or in other liquid crystals [3]. Here, we show that this methodology can be extended to determine a sufficient amount of dipolar couplings in order to determine the structure and also relative stereochemistry of moieties in menthol. The investigation of conformation and configuration of organic molecules by the study in liquid crystals using dipolar couplings has been pioneered by seminal work of Emsley and Lindon [4]. However, at the time, the NMR methodology was not developed to the degree it is now. This manuscript shall contribute to make use of dipolar couplings as much as possible. We acknowledge related but independent work in the same direction by Thiele and Berger [5].

It is by now well established that homo- and heteronuclear dipolar couplings of molecules dissolved in aqueous liquid crystals improve the structure determination through the introduction of orientational restraints [6]. In addition, dipolar couplings can also be used to better define the dynamics of proteins in solution [7]. However, this approach could not easily be applied to molecules that are insoluble in water. Here, we use poly-γ-benzyl-glutamate in chloroform to measure dipolar couplings of menthol, introduce a new robust method for the measurement of long range H,C coupling constants and demonstrate that the dipolar couplings can be used to obtain stereochemical assignments of diastereotopic groups. This is based on the fact that for each stereocenter the measurement of five independent dipolar couplings allows to determine its orientation in the molecule (Fig. 1). Since dipolar couplings are inversion symmetric they alone are insufficient to make the distinction between epimers. However, dipolar couplings allow only certain orientations of each stereocenter in the molecule between which then *J*-couplings and NOEs will distinguish with much higher accuracy than if this information is not available. We apply this method to menthol whose structure is known both from X-ray crystallography as well as from solution NMR.

The liquid crystal is prepared as already published in [2]. PBLG obtained from Sigma with a specified

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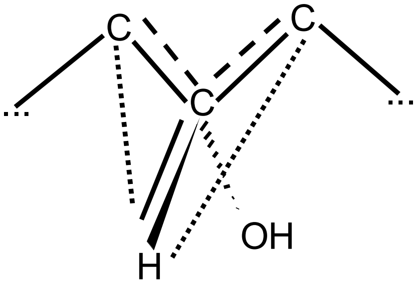

Fig. 1. Stereocenter with two inequivalent carbon ligands, one proton and one non-NMR active nucleus. In order to determine the stereochemistry of this center, three dipolar couplings can be measured and five, if the concentration allows for C,C coupling measurements per alignment medium. The ${}^1D_{CH}$ (continuous line), two ${}^2D_{CH}$ (dotted line), and ${}^1D_{CC}$ (dashed line) couplings are sufficient to determine the orientation of a stereocenter in space except for inversion. This requires two alignment media in the case that C,C couplings are not available.

molecular weight of 150–350 kDa induces a large degree of alignment in chloroform solutions. We measured deuterium splitting of chloroform of around 200 Hz depending on the amount of chloroform added. The alignment was sufficiently small to allow proton detection. Several steps of centrifugation about the rotation axis of the NMR tube are crucial to obtain a homogeneous nematic phase. Shimming using either resonance of the $CDCl_3$ lock signal turned out to be inferior compared to shimming on the DMSO lock signal produced by a capillary containing d_6-DMSO.

One-bond dipolar couplings ${}^1D_{CH}$ are measured in the proton dimension by recording an HSQC experiment without proton decoupling during the detection period. By contrast to the conventional method to measure the splitting in ω_1 the measurement in ω_2 allows to measure C,H couplings also in CH_2 groups for both protons individually. A representative section of the spectrum is shown as well as traces through cross peaks in the isotropic and the anisotropic spectrum in Fig. 2. The coupling constants determined are listed in Table 1. Due to the sufficiently fast rotation of the methyl group about the axis of the carbon to the next heavy atom the ${}^1D_{CH_3}$ depends on the orientation of the vector between the methyl carbon and the directly bound heavy atom. Therefore, we express these ${}^1D_{CH_3}$ values in terms of ${}^1D_{CC}$ couplings according to ${}^1D_{CC} = {}^1D_{CH_3}(-3\gamma_C/\gamma_H)(r^3_{CH}/r^3_{CC})$, where γ_C and γ_H are the gyromagnetic ratios of carbons and protons and r^3_{CH} and r^3_{CC} are the C,H and C,C distances. The couplings have been determined by shifting the low field and the high field submultiplet components on top of each other. The error is approximately 0.5 Hz for the dipolar couplings and 0.3 Hz for scalar couplings.

C,C coupling constants have been measured by a conventional C,C INADEQUATE spectrum [8] and are listed in Table 1. Representative sections and traces in isotropic and anisotropic solution are shown in Fig. 3.

For the measurement of long range H,C dipolar couplings we propose a modified HMBC experiment that builds on the approach by Keeler and Neuhaus [9] where a 1D reference spectrum is used to compare it with traces from the HMBC experiment. While this approach works very well in cases of completely resolved 1D ${}^1H$ NMR spectra it fails in cases of overlap in the 1D proton spectrum. Therefore, we measured as a reference experiment a modified HSQC experiment that reproduces the transfer amplitude of the HMBC except for the two sine factors due to the defocusing and refocusing of the active long range coupling constant. The pulse sequence is given in Fig. 4. The transfer amplitude for the HMBC between the proton H and the long range carbon C_n is described as:

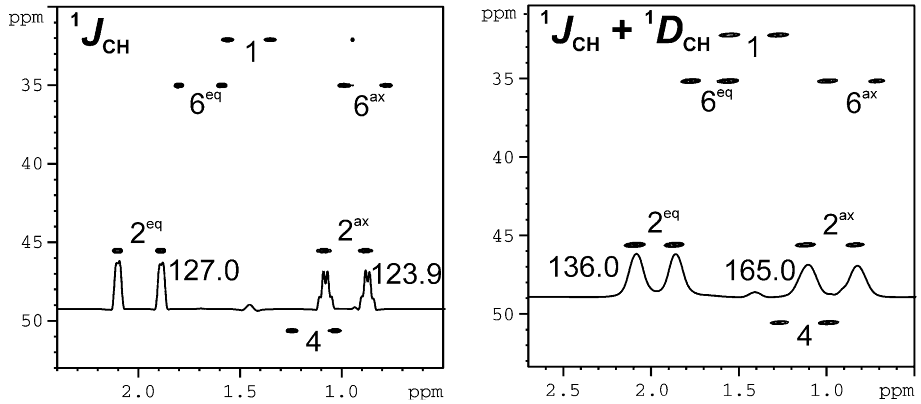

Fig. 2. Sections of HSQC spectra and traces through the HSQC spectra recorded on a 100 mM menthol sample at 303 K without decoupling in t_2. Left, in $CDCl_3$ solution and right after addition PBLG. Both experiments were recorded at 600 MHz using 16 scans, 8 K points in the acquisition domain and a spectral width of 6009 Hz in ω_2. Two hundred fifty-six complex experiments for t_1 are recorded with a spectral width of 10,416 Hz in ω_1. The splitting extracted from the isotropic and anisotropic solutions represent the ${}^1J_{CH}$ scalar coupling and ${}^1J_{CH} + {}^1D_{CH}$ dipolar couplings, respectively. The splittings have been extracted by fitting the line shapes of the low-field and high-field lines.

Table 1
Experimental short and long range couplings

	Scalar couplings (Hz)	Dipolar couplings (Hz)
^1J		
Carbon–proton		
C_1–H_1	125.4	41.5
C_2–H_2^{eq}	127.0	9.0
C_2–H_2^{ax}	123.9	41.1
C_3–H_3	139.8	41.4
C_4–H_4	124.9	43.8
C_5–H_5^{eq}	127.5	5.3
C_5–H_5^{ax}	124.7	39.8
C_6–H_6^{eq}	124.4	4.7
C_6–H_6^{ax}	126.5	38.6
C_8–H_8	126.5	8.5
Carbon–carbon		
C_1–C_2	32.3	−0.7
C_2–C_3	36.0	−4.5
C_3–C_4	36.7	0.5
C_4–C_5	33.0	−0.4
C_5–C_6	33.0	−4.5
C_6–C_1	33.4	0.3
C_1–C_7	35.9	−5.3
C_8–C_9	35.6	0.8
C_8–C_{10}	35.2	2.9
C_4–C_8	35.2	−5.1
^2J		
C_4–H_8	7.6	−2.0
C_2–H_1	1.8	1.2
C_2–H_3	1.4	−1.2
C_4–H_3	1.4	1.2
C_1–H_2^{eq}	3.6	−0.2
C_5–H_4	6.0	3.5
^3J		
C_4–H_2^{eq}	6.0	−0.8
C_5–H_3	1.2	1.9
C_5–H_8	7.4	−2.2
C_6–H_2^{eq}	6.8	0.7
C_1–H_3	1.2	−1.0
C_3–H_1	1.4	−1.0
C_7–H_2^{eq}	1.6	1.2
C_8–H_3	1.8	−1.3
C_8–H_5^{eq}	1.8	−1.0

Scalar couplings are only mentioned when dipolar couplings were derived.

$$p_{C_n} \sin(\pi\, {}^nJ_{C_nH} \varDelta) \prod_{i=1}^{N} \cos\left(\pi J_{H_iH}(\varDelta + t_1 + t_2)\right) \sin\left(\pi\, {}^nJ_{C_nH} t_2\right),$$

where p_{C_n} is the abundance of C_n, the carbon in question, ${}^nJ_{C_nH}$ is the long range scalar coupling and includes the ${}^nD_{C_nH}$ dipolar coupling in anisotropic medium.

By contrast, the reference HSQC experiment provides the following transfer amplitude:

$$p_C \sin(\pi\, {}^1J_{CH} \varDelta) \prod_{i=1}^{N} \cos\left(\pi J_{H_iH}(\varDelta + t_1 + t_2)\right) \sin\left(\pi\, {}^1J_{CH} t_2\right),$$

p_C is the natural abundance of the carbon directly bound to the proton. The number of relevant pulses and effects of relaxation have been taken into account as much as possible. Assuming that the natural abundances of the two carbons are the same and given the fact that the coupling constant ${}^1J_{CH}$ can be directly read off from the HSQC reference spectrum, both the amplitude and the line shape of the HMBC peak can be fitted by variation of only the ${}^nJ_{C_nH}$ coupling constant. The better resolution of the HSQC derived experiment compared to a 1D NMR reference spectrum constitutes an improvement over the Keeler Neuhaus sequence in which both amplitude and splitting were fitted. Of course, the reference experiment works only for carbon bound protons. Overlap of signals in the HMBC or the reference experiment also disturb the fitting. For this reason, multiplets that are too close to methyl signals were not fitted (Table 1). The correct value of $J(\mathrm{H},\mathrm{C})$ is the one that minimizes the difference between the ω_2-multiplet pattern of the HMBC and the reconstructed antiphase multiplet from the reference spectrum (Fig. 5).

Experimental long range isotropic couplings as well as dipolar couplings are listed in Table 1. The fitting procedure is shown in the example of the H_3–C_1 cross peaks of menthol putting the high accuracy of the method into clear evidence (Fig. 6). The dipolar couplings compiled in Table 1 were used to determine the alignment tensor with the program PALES [10]. This approach not invoking any assumption about the alignment tensors requires a single major conformation of the molecule.

Using all dipolar couplings reported in Table 1 the magnitude and rhomibicity of the alignment tensor expressed in terms of ${}^1D_{NH}$ dipolar couplings are: $D_a^{NH} = -14\,\mathrm{Hz}$, $R = 0.3$. The alignment is mainly steric [10], the main axis passing approximately through C_1 and C_4. Fig. 7 demonstrates that observed dipolar couplings fit very well to dipolar couplings back-calculated from the X-ray structure of menthol. The linear curve fitting of D_{calc} to D_{obs} is $D_{calc} = D_{obs} - 0.02\,\mathrm{Hz}$ with a Pearson's correlation factor $R^{(2)}$ of 0.996. The high correlation and small offset of 0.02 Hz puts the high accuracy of the structure and dipolar couplings into evidence.

In order to evaluate the possibility of determining the stereostructure we performed three tests: The first test refers to inversion of the stereocenter at C_1. We changed the configuration 1R to 1S and then calculated the tensor. The involved couplings at the C_1 stereocenter are ${}^1D_{C_1H_1} = 41.5\,\mathrm{Hz}$ and ${}^1D_{C_1C_7} = -5.3\,\mathrm{Hz}$. The latter ${}^1D_{CC}$ coupling is equivalent to a ${}^1D_{CH}$ dipolar coupling along the C_1–C_7 bond of ${}^1D_{CH} = -59.3\,\mathrm{Hz}$. Thus, we expect a very clear distinction between the two possible stereochemical assignments. Indeed, the correlation between D_{calc} and D_{obs} was dramatically decreased upon epimerisation, the new linear fitting gave a correlation factor $R^{(2)}$ of 0.780 and a fit $D_{calc} = 0.72D_{obs} + 0.1$ (data not shown). This demonstrates the ability of dipolar couplings to discriminate R or S configurations when the geometry is fixed.

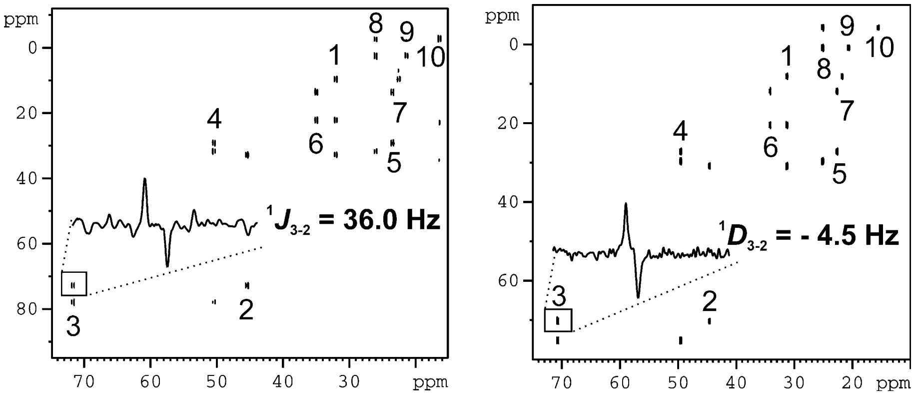

Fig. 3. C,C-INADEQUATE experiment on 2 M menthol at 303 K. Left, in $CDCl_3$ solution and right after addition of PBLG. The spectrum of the isotropic solution has been recorded at a proton frequency of 400 MHz while the anisotropic one has been recorded at 600 MHz using a TXI-cryoprobe. The C,C couplings can be directly read off. We used 512 scans, 4 K points in t_2 and 128 experiments in t_1. The spectral width was 18181.82 Hz (double quantum frequency) in ω_1 and 9057 Hz in ω_2. The splittings have been extracted by fitting the line shapes of the low-field and high-field lines.

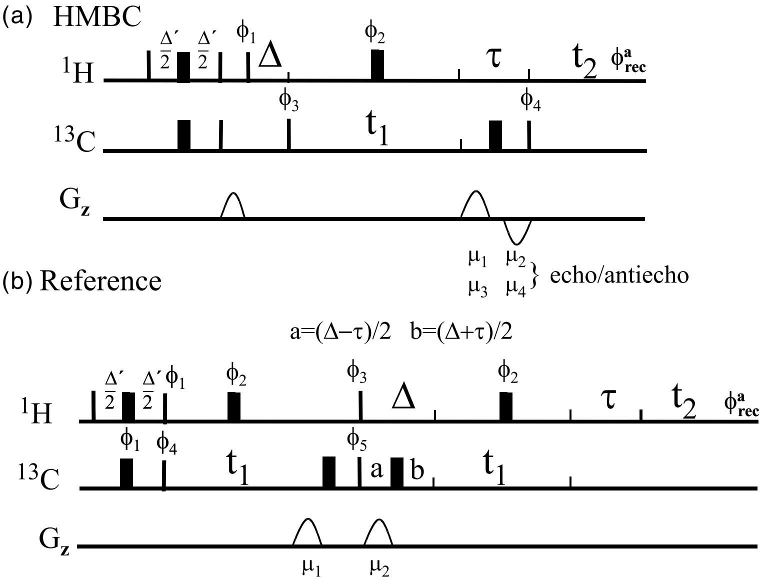

Fig. 4. Pulse sequence of the HMBC and the reference HSQC experiment. Both sequences yield the same multiplet structure in ω_1 and ω_2 except for the heteronuclear long range coupling as described in the text. (a) HMBC: narrow bars, 90° pulses; wide bars, 180° pulses, phase is x if not specified otherwise. Echo and antiecho selections were achieved using gradients. The carbon pulse phase ϕ_3 was incremented by π for each t_1 increment to obtain States-TPPI-like data. $\phi_1 = (+x)_4(-x)_4$; $\phi_2 = (+x)_8(+y)_8$; $\phi_3 = +x - -x$; $\phi_4 = +x + x - x - x$; $\phi^a_{rec} = \phi_1 + 2\phi_2 + \phi_3 + \phi_4$. Values for τ = 2.84 ms and Δ = 20 ms are used for the HMBC experiment. Gradient lengths are 1 ms with a recovery delay of 400 μs and gradient settings were [echo: $(\mu_1, \mu_2) = (+50, -30)$; antiecho: $(\mu_3, \mu_4) = (+30, -50)$. (b) Reference HSQC: $\phi_1 = +y, +y, -y, -y$; $\phi_2 = (+x)_{16}(-x)_{16}$; $\phi_3 = (+x)_8(-x)_8$; $\phi_4 = +x - x$; $\phi_5 = (+x)_4(-x)_4$; $\phi^a_{rec} = \phi_1 + \phi_3 + \phi_4 + \phi_5$. Values for the delays are the same as in the HMBC, Δ' is set to 3.4 ms. In (b) gradient settings are: $(\mu_1, \mu_2) = (\pm 40, +10)$ for echo/antiecho selection.

Although chemically not so interesting, the distinction between chirality of stereocenters and the correct assignment of prochiral groups pose equal challenges. The two other tests therefore are applied to the distinction of two diastereotopic pairs. First, we compared the fitting result when the two diastereotopic protons H_2^{eq} and H_2^{ax} are interchanged (Fig. 8). With 9.0 and 41.1 Hz the experimental dipolar couplings differ significantly for the two protons. The back-calculated alignment tensor changes upon interconversion of the two couplings and the overall fit becomes substantially worse. Using the correct stereospecific assignment observed and back-calculated $^1D_{CH}$ couplings agree very well with $R^{(2)} = 0.995$ and $D_{calc} = D_{obs} - 0.1$ Hz while after interchange $R^{(2)} = 0.670$ and $D_{calc} = 0.7D_{obs} + 5.31$ Hz. Using all dipolar couplings listed in Table 1 we

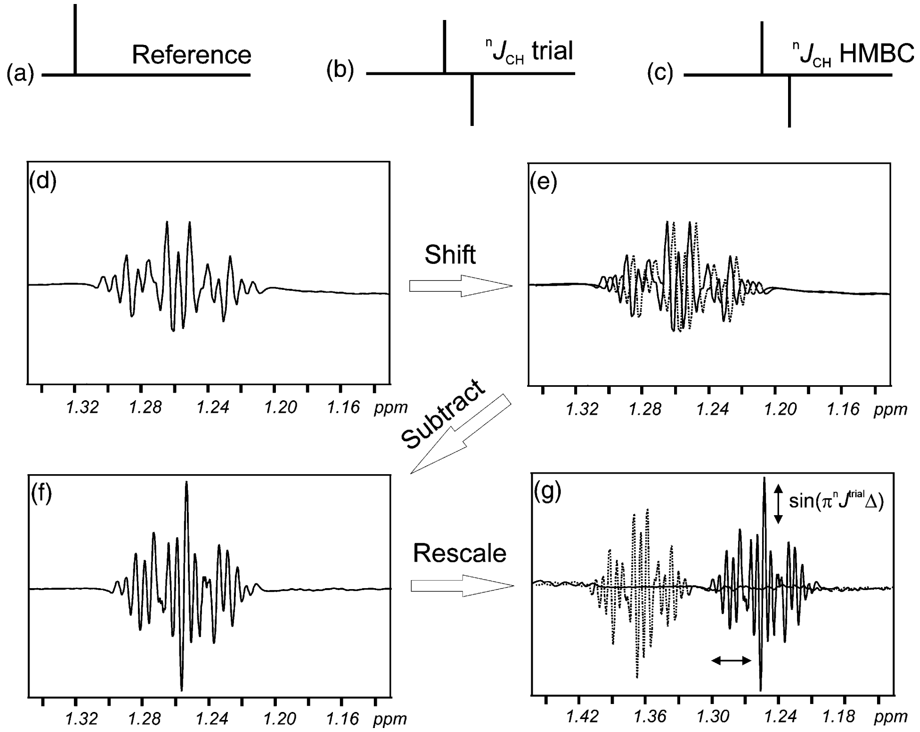

Fig. 5. Extraction of the ${}^{n}J_{CH}$ coupling from the HMBC experiment and the reference experiment as described in Fig. 4 applied to 1 M menthol in chloroform. An ω_2-trace of the reference experiment (a) is duplicated and shifted by the trial ${}^{n}J_{CH}$ coupling and subtracted from the original trace (b). The result is scaled by $\sin(\pi\, {}^{n}J_{CH}\Delta)$ and compared with the HMBC trace (c). This procedure has only one fitting variable and is therefore very precise. Traces (d)–(g) are the reference spectrum (d), the duplicated and shifted reference spectrum (e), the subtracted spectrum (f) and the fitting between the subtracted spectrum and the HMBC multiplet (dotted line) (g).

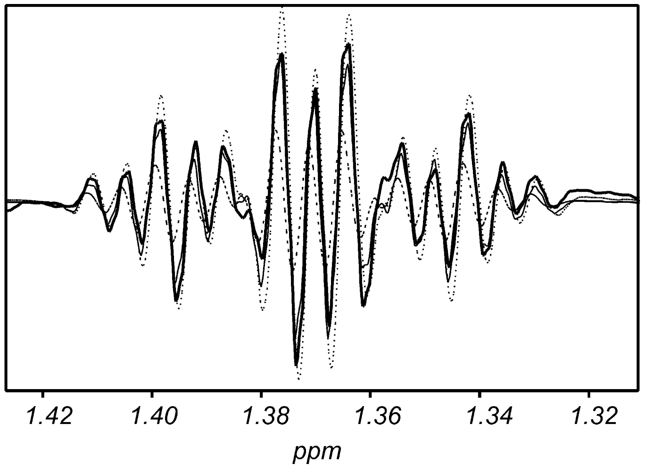

Fig. 6. Fitting result for the cross peak between H_3 and C_1 in isotropic solution. The row extracted from the HMBC is bold. The precision is put into evidence from the traces reflecting 1 Hz (dashed), 1.4 Hz (thin, best match), and 1.8 Hz (dotted).

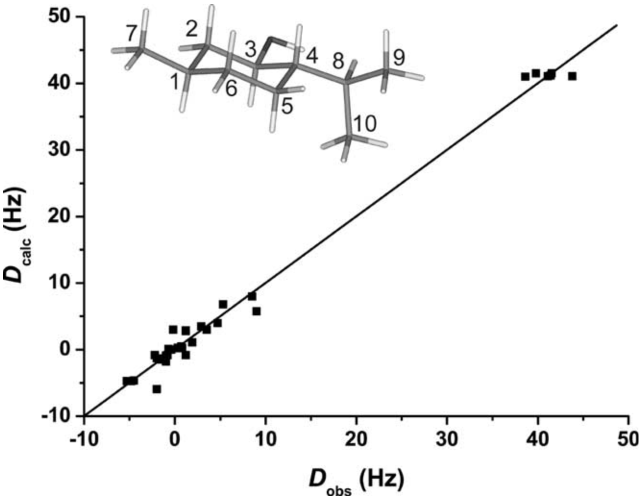

Fig. 7. Calculated and experimental dipolar couplings for all couplings of Table 1 between directly and indirectly bound H,C pairs as well as directly bound C,C pairs. The X-ray structure used for calculations was first minimized using conjugate gradients. The observed dipolar couplings match very well with the ones back calculated from the X-ray structure with a correlation coefficient $R^{(2)} = 0.996$.

find: $R^{(2)} = 0.996$ with $D_{calc} = D_{obs} - 0.02\,\text{Hz}$ while after interchange $R^{(2)} = 0.92$ and $D_{calc} = 0.88D_{obs} + 0.06\,\text{Hz}$.

A more challenging example is the simultaneous definition of the stereochemistry of the two methyl groups C_9 and C_{10} and their conformation using anisotropic restraints derived from dipolar couplings and isotropic restraints derived from NOEs and ${}^{3}J_{HH}$. Stereochemistry and conformation need to be determined since the C_4–C_8 bond is rotatable. We varied the torsion

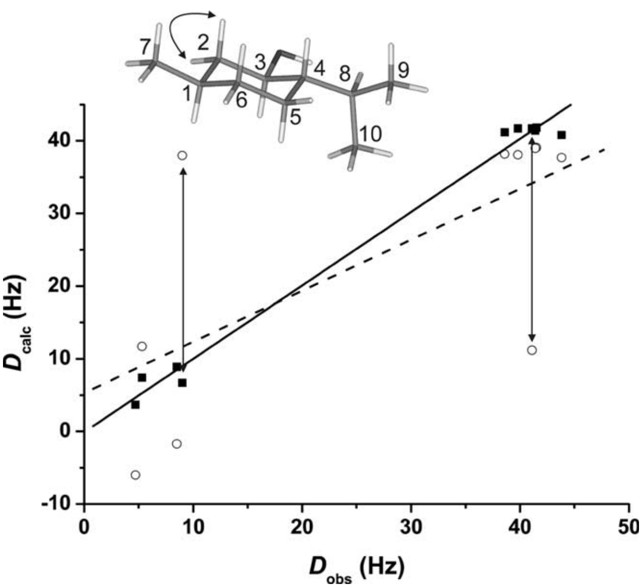

Fig. 8. Superimposition of the fitting curves when diastereotopic protons are exchanged. Dashed curve and circles are used for the wrong diastereotopic assignment and show that exchange of the two diastereotopic protons at the C_2 position yields a much worse fit considering only the $^1D_{CH}$ couplings.

angle about the C_4–C_8 bond in steps of 20° with both possible stereoassignments of C_9 and C_{10}. For each conformation and stereoassignment alignment tensors are calculated from all dipolar couplings of Table 1 using singular value decomposition (SVD) [11] as implemented in the PALES software. As quality factor, we calculated the RMS ($RMS = \sqrt{\sum(D_{calc} - D_{obs})^2}$) for $^1D_{C_8H_8}$, $^1D_{C_8C_9}$, and $^1D_{C_8H_{10}}$ as a function of the dihedral angle H_4–C_4–C_8–H_8 for the two possible stereoassignments. The two different stereoassignments are called **1** with H_9 downfield and H_{10} upfield and **2** with the opposite assignment. The union (g^+, **2**) is the conformation g^+ and the assignment is that of **2**. Only 9 unions displayed a low RMS value (<1.5 Hz). To differentiate between these unions we used the $^3J_{H_4H_8}$ coupling of 2.6 Hz as supplementary restraint leaving the following unions: ($-110°$, **1**) with RMS = 1.10 Hz, ($-110°$, **2**) with RMS = 0.82 Hz, ($-60°$, **1**) with RMS = 0.60 Hz, (60°, **2**) with RMS = 1.32 Hz, and (110°, **2**) with RMS = 1.25 Hz. Out of these conformations, the ($-60°$, **1**) has the lowest RMS value by a factor of at least 1.5. To check whether the RMS deviation between the different unions was reproducible, we repeated the tensor calculations adding in one case all $^1D_{CC}$ and in another case adding $^1D_{CC}$, $^2D_{CH}$ as well as $^3D_{CH}$. The RMS values obtained allow for a clear assignment of the correct union ($-60°$, **1**): 1.12 Hz ($-110°$, **1**), 0.84 ($-110°$, **2**), 0.50 Hz ($-60°$, **1**), 0.94 Hz (60°, **2**), and 1.06 Hz (110°, **2**) for the first case and 1.32 Hz (110°, **1**), 1.50 Hz (110°, **2**), 0.89 Hz (60°, **1**), 1.46 Hz (60°, **2**), and 1.20 Hz (110°, **2**) for the second one.

In conclusion, we have shown that it is possible to measure H,C, C,C, and long range H,C dipolar couplings in organic molecules that are water insoluble. We have introduced a new robust HMBC method that requires only one fitting parameter, works for overlapped proton 1D NMR spectra, and provides 2D resolution also in the reference experiment. It was possible to assign one chiral center correctly and to distinguish diastereotopic groups in rigid as well as conformationally flexible parts of the molecule. The combined use of direct H,C, direct C,C, and long range H,C dipolar couplings has the potential to define better the stereochemistry of organic compounds when dissolved in organic solvents. The C,C dipolar coupling measurement is only possible using a high concentration of compound. However, in case of methyl groups the D_{CX} coupling between the methyl and the heavy atom X directly bound to it can be easily back-calculated from $^1D_{CH_3}$ alleviating at least the measurement of the C,C couplings to methyl group. We will in the future explore this possibility to solve questions of relative stereochemistry in organic compounds.

Acknowledgments

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